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EARLY CHANGES OF MUSCLE IGF-1 AND MYOSTATIN GENE EXPRESSION IN GASTRIC CANCER PATIENTS

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Running title: Myostatin and IGF-1 in gastric cancer

Abstract

Introduction: Cachexia is a devastating condition that increases morbidity and mortality rates in cancer patients. It often becomes evident only at advanced disease stages, when responsiveness to currently available treatments may be limited or even absent. The progressive loss of muscle mass is by far the most prominent feature of cancer cachexia, negatively affecting physical function and quality of life. We previously reported that reduced IGF-1 expression and enhanced myostatin signaling in the muscle of tumor-bearing animals. The present study was aimed at investigating whether similar perturbations occur in gastric cancer patients as well.

Methods: Early perturbations of myostatin and IGF-1 signaling (including the expression of muscle-specific ubiquitin-ligases) were investigated in 16 gastric cancer patients and in 6 control subjects analyzing muscle mRNA expression by both semi-quantitative RT-PCR and real-time PCR.

Results: In gastric cancer patients, muscle mRNA levels for IGF-1, myostatin and atrogin-1 were reduced while MuRF1 expression was unchanged. Changes occurred irrespective of weight loss ($\leq 5\%$ or $> 5\%$).

Discussion: The observation that IGF-1 and myostatin mRNA levels are down-regulated in gastric cancer patients with minimal or no weight loss indicates that molecular alterations in the skeletal muscle should be regarded as an early event in the course of the disease. This is particularly relevant in order to devise preventative and therapeutic strategies for cancer cachexia.

Key words: IGF-1; myostatin; ubiquitin ligases; human cancer; cachexia.

Introduction

Patients with neoplastic disease frequently develop cachexia, a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) and alterations of energy balance, driven by a variable combination of reduced food intake and abnormal metabolism. Conventional nutritional support cannot fully reverse this life-threatening pathological condition.¹ Some of the causative factors had been identified, although the mechanisms underlying muscle protein hypercatabolism, one of the most important features of cachexia, still remain elusive. In particular, most of the information available about the pathogenesis of cachexia are derived from experimental studies, and very little is directly known about human cancer. Moreover, since cachexia often becomes clinically evident at a rather advanced disease stage, the detection of early subclinical perturbations that may be indicative of a situation of 'pre-cachexia'^{2,3} is important in order to take appropriate preventive measures to prevent the onset of overt cachexia.

Studies performed in the last decade led to unravel signaling pathways potentially contributing to the onset of muscle wasting. Particularly interesting, in this regard, are those regulated by Insulin-like growth factor (IGF)-1 and myostatin. The former is an anabolic growth factor known to stimulate protein synthesis rates in the skeletal muscle, as well as to induce satellite cells to proliferate and differentiate.⁴ Inhibition of apoptotic cell death and suppression of proteolysis, mainly due to down-regulation of the ubiquitin-proteasome system are some of the effects exerted by IGF-1 on muscle tissue.⁵ In this regard, reduced IGF-1 circulating levels were reported in catabolic states such as cancer and sepsis.⁶⁻⁸

Myostatin has been proposed to negatively regulate skeletal muscle mass.⁹ Muscle hypertrophy in cattles and humans has been reported to be associated with myostatin inactivating mutations.^{10,11} Consistently, increased muscle mass develops in mice when myostatin gene is disrupted, or the levels of active myostatin are low. Similarly,

overexpression of a dominant negative form of the myostatin receptor ActRIIB, or administration of soluble ActRIIB lead to muscle hypertrophy.^{12,13} By contrast, high myostatin levels result in extensive skeletal muscle depletion.^{13,14} Myostatin protein levels are increased in ageing, denervation atrophy or mechanical unloading,¹⁵ while reduced in the regenerative response induced in muscles by bupivacaine or notexin.^{16,17} Results from our laboratory demonstrate that myostatin signaling is enhanced in the skeletal muscle of tumor-bearing rats and mice^{18,19} and consistently, myostatin inhibition by antisense oligonucleotides, by administration of an ActRIIB/Fc fusion protein or and ActRIIB soluble form prevent muscle wasting in tumor-bearing mice.²⁰⁻²²

Aim of the present study was to investigate whether early molecular perturbations occur in the skeletal muscle of gastric cancer patients. For this purpose, gene expression of myostatin, IGF-1 and muscle-specific ubiquitin ligases atrogen-1 and MuRF1 was evaluated.

Material and methods

Patients

Sixteen patients with gastric cancer admitted to the Department of Surgery, Second Faculty of Medicine, 'Sapienza' University of Rome, Italy, were included. The study was approved by the local ethical committee and written informed consent was obtained from all patients before enrolment in the study. Diagnosis of gastric cancer was made by endoscopic biopsy. Patients undergoing abdominal surgery for non-neoplastic diseases (inguinal hernia, laparocoele, abdominal aorta aneurysm) served as controls (n = 6). Exclusion criteria for both groups were: liver failure, diabetes, acute or chronic renal failure, metabolic acidosis, AIDS, inflammatory bowel disease, autoimmune disorders, sepsis, chronic obstructive pulmonary disease, chronic heart failure, hepatitis, hyperthyroidism. Some of the subjects enrolled in the present study also participated in a

previous investigation from our group.²³ After an overnight fast, subjects were studied at 8:00 am. Nutritional assessment was performed by evaluating anthropometric parameters, usual body weight, percent weight loss, biochemical and immunological indices (see Table 1).

Muscle biopsy

A biopsy specimen was obtained from the *rectus abdominis* muscle during the initial phase of the operation. The anterior sheet of the *rectus abdominis* muscle was opened with scissors after skin incision and dissection through the subcutaneous fat, and a muscle biopsy specimen weighing about 0.5 g was obtained. The biopsy specimen was immediately frozen in liquid nitrogen and then stored at -80°C until analysis. Small bleeding vessels were carefully controlled with ligatures and cautery after the muscle biopsy had been obtained, whereafter the operation continued in a routine fashion. No complications occurred from the biopsy procedure.

Reverse Transcription-PCR

Total RNA was extracted using the TriPure reagent (Roche, Indianapolis, IN, USA) and following manufacturer's instructions. RNA concentration was determined spectrophotometrically (260nm), purity ensured by 260/280nm ratio, and integrity checked by electrophoresis on 1.2% agarose gel, containing morpholino propane-sulfonic acid (MOPS) 0.02 M and 18% formaldehyde (37%).

mRNA levels of the analytes were determined by semiquantitative RT-PCR using the kit 'Ready-to-Go' (GE Healthcare, Milano, Italy). Primers for GAPDH, IGF-1, Myostatin, Atrogin-1 and MuRF1 were obtained according to published sequences (Table 2; Invitrogen, Milano, Italy). Retrotranscription was performed at 42°C for 30 min. Amplification was run as specified in Table 2. Positive and negative controls were performed. PCR products (GAPDH = 497bp; IGF-1 = 184bp; Myostatin = 79bp; Atrogin-1 = 168bp; MuRF-1 = 225bp) were electrophoresed on 2% agarose gels and visualized by

staining with ethidium bromide. A 100bp-standard DNA ladder (Fermentas, Burlington, ON, Canada) was used to estimate PCR products length. Quantification was performed by densitometric analysis: individual product bands and representative background were excised from each gel lane and analyzed by means of a specific software (Phoretix). The results were normalized according to GAPDH expression (conditions of amplification reported in Table 2). Groups were compared in the linear range of amplification.

As for real-time PCR, a new RNA extraction was performed as reported above. RNA was quantified fluorimetrically using the Quant-iT RiboGreen reagent (Molecular Probes, Eugene, OR, USA). 0,5 µg of total RNA were retro-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Transcript levels were determined by using the SsoFast Evagreen Supermix and the MiniOpticon thermal cycler (Bio-Rad). 10 seconds of denaturation at 95 °C were followed by 30 seconds of annealing/extension at 60 °C and repeated for 40 cycles. Gene expression was normalized to both GAPDH and TBP expression (see Table 2 for primer sequences).

Data analysis and presentation

Results are expressed as mean \pm SEM. Significance of the differences was evaluated by Student's unpaired 't' test corrected according to Welch, as well as by a non-parametric test (Mann-Whitney), and were considered significant at $p < 0.05$. Data obtained from the two tests were comparable.

Results

Subject characteristics are shown in Table 1. Mean age was 64 ± 2.75 years for patients and 62 ± 5.71 years for controls (data expressed as means \pm SEM). Body weight loss $<5\%$ was detectable in 8 out of the 16 patients (50%), while median weight loss of the whole group ($n = 16$) was 5.45% (see ref. 2). As for clinical parameters, only haemoglobin

levels were significantly decreased in cancer patients with respect to control values (Table 1). Such reduction did not correlate with body weight loss and muscle gene expression.

IGF-1 mRNA expression in the skeletal muscle of gastric cancer patients was markedly reduced (70% of controls; $p < 0.05$; figure 1A), even more when real-time PCR was used (controls = 1 ± 1.693 , patients = 0.62 ± 1.46 ; normalized fold expression, $p < 0.05$; figure 1B). The reduction was maintained also when patients were stratified according to weight loss (<5%: 72% of controls; >5%: 76% of controls; performed on RT-PCR data), while IGF-1 expression was comparable in stage I-II or III-IV patients (stage I-II: 0.34 ± 0.03 , $n = 12$; stage III-IV: 0.36 ± 0.02 , $n = 4$).

Muscle myostatin mRNA expression was markedly and significantly ($p < 0.001$) reduced with respect to control subjects (figure 2A and 2B), irrespective of body weight loss (<5%: 49% of controls; >5%: 47% of controls). Also for myostatin, the reduction observed in real-time PCR is more marked (controls = 1 ± 1.79 , patients = 0.30 ± 1.18 ; normalized fold expression, $p < 0.001$; figure 2B). No differences were observed when patients were stratified according to tumor stage (stage I-II: 0.46 ± 0.04 , $n = 12$; stage III-IV: 0.37 ± 0.01 , $n = 4$). In addition, no correlation with IGF-1 expression was observed (Spearman test: $r = 0.336$, $p = 0.240$).

Gene expression for both atrogin-1 and MuRF1 in gastric patients and controls is shown in figure 3. Atrogin-1 expression was 45% of controls ($p < 0.001$). Such observation, however, was not confirmed by real-time PCR, that revealed only a tendency to reduction, not statistically significant (controls = 1 ± 1.41 , patients = 0.74 ± 1.35 ; normalized fold expression, $p = 0.817$). As for MuRF1, no significant differences were observed between controls and cancer patients, although real-time PCR revealed a tendency to increase that did not reach significance (controls = 1 ± 1.23 , patients = 1.42 ± 1.27 ; normalized fold expression, $p = 0.296$). Stratification according to body weight loss lead to comparable results for both ubiquitin ligases (atrogin-1: <5% = 49% of controls, >5% = 45% of controls;

MuRF1: <5% = 118% of controls, >5% = 112% of controls). Ubiquitin ligase expression was not changed in stage I-II compared with stage III-IV patients (Atrogin-1: stage I-II, 0.41 ± 0.04 , $n = 12$; stage III-IV, 0.51 ± 0.08 , $n = 4$. MuRF1: stage I-II, 0.38 ± 0.01 , $n = 12$; stage III-IV, 0.36 ± 0.03 , $n = 4$).

Discussion

The results obtained in the present study show that, similarly to experimental cachexia, IGF-1 mRNA expression is significantly reduced in the skeletal muscle of gastric cancer patients.

This is the first study reporting that IGF-1 mRNA levels are decreased in the muscle of cancer patients, supporting the hypothesis that a down-regulation of the IGF-1 signaling is involved also in the pathogenesis of human cancer cachexia. Interestingly, such reduction is detectable either when patients are pooled together, or when they are stratified according to body weight loss (<5% or >5%). This is an important finding since it further confirms that observations made in experimental models may well apply to human cancer. Moreover, similar observations showing a marked down-regulation of IGF-1 and IGF-binding proteins 3, 4 and 5 despite an increase in IGF-1R expression have been reported in a study conducted on patients affected by sporadic amyotrophic lateral sclerosis²⁴. However, such a view cannot be generalized. Indeed, the expression levels of muscle myostatin mRNA, which have been shown to be enhanced in tumor-bearing animals, are significantly reduced in gastric cancer patients. This result appears particularly surprising as we previously reported an increase in myostatin protein level in gastric cancer patients.²³ Although the reason for these apparently contradictory results is not known at present, the differences may at least in part be due to post-transcriptional mechanisms such as increased myostatin synthesis secondary to increased translational efficiency or reduced degradation of myostatin. Interestingly enough a similar discrepancy

between myostatin mRNA and protein levels have been previously reported in other conditions associated to muscle atrophy.^{25,26} It should be noted, however, that most of the patients included in the present study were in disease stages 1 or 2. It cannot be excluded that myostatin perturbations may become apparent in more advanced stages of the disease when more significant weight loss and cachexia occur. Further research in this respect is warranted. Finally, reduced myostatin mRNA might reflect an early compensatory response activated by the muscle to maintain the homeostasis.

Previous results from our laboratories showed that hyperactivation of the ATP-ubiquitin-dependent proteolytic system is an early event in the skeletal muscle of gastric cancer patients. Indeed, both ubiquitin mRNA expression and proteasome enzymatic activities were found significantly elevated before any evidence of body weight loss.^{23,27} In the present study the expression of muscle-specific ubiquitin ligases atrogin-1 and MuRF1 was assessed. As for IGF-1 and myostatin, also atrogin-1 and MuRF1 expression was evaluated by both RT-PCR and real-time PCR. While both techniques gave qualitatively comparable results for IGF-1 and myostatin, the data obtained for atrogin-1 and MuRF1 were slightly different. Indeed, the significant reduction of atrogin-1 mRNA levels when evaluated by RT-PCR was no more evident by real-time PCR, although a tendency to decrease occurred. Similarly, MuRF1 expression was unchanged in cancer patients with respect to controls, whether evaluated by RT-PCR or by real-time PCR, although the latter showed a tendency to increase. On the whole, our conclusion is that muscle-specific ubiquitin ligase gene expression is not significantly modified in cancer hosts, at least in the present group of patients. Such observations agree with those reported by a previous study showing that atrogin-1 and MuRF1 gene expression in gastric cancer patients was not significantly affected, despite a trend toward increase occurred.²⁸

The reasons for these inconsistencies remain so far unknown, underscoring the need for further studies aimed at clarifying whether increased mRNA expression for

ubiquitin ligases may really be considered a molecular marker of muscle atrophy also in human diseases associated with muscle loss. In this regard, muscle atrophy in patients affected by spinal cord injury is associated with low expression of IGF-1, MuRF1 and atrogin-1.²⁹ However, it cannot be excluded that in human cancer cachexia a significant increase in atrogin-1 and MuRF1 mRNA levels may occur with greater rates of weight loss. This hypothesis is supported by the observation that reduced expression of proteasome subunits was not apparent in cancer patients with weight loss < 10%.³⁰ Indeed gastric cancer patients included in the present study presented only minimal or no weight loss.

Interestingly, in the present study, no increase of atrogin-1 mRNA levels could be detected, either by RT-PCR or by real-time PCR. Although this finding is somehow surprising, it should be noted that pharmacological correction of muscle wasting in cancer cachexia is not always paralleled by changes in atrogin-1 expression.^{7,31} This observation suggests that changes of muscle mass and atrogin-1 mRNA levels might not be tightly coupled. This hypothesis is also consistent with another report showing that restoration of normal atrogin-1 expression induced in fasted or diabetic rats by IGF-1 treatment was not associated with muscle mass recovery.³² In addition, reduced protein degradation rates resulting from incubation of muscles isolated from burned rats in the presence of GSK3- β inhibitors was reported to occur irrespective of changes in atrogin-1 mRNA levels.³³ Finally, there are no available reports demonstrating a correlation between ubiquitin ligase mRNA levels and proteasome enzymatic activity. Based on these observation, Attaix and Baracos recently proposed that “atrogin-1 expression is a poor index of muscle proteolysis”.³⁴

In conclusion, although the sample size might represent a limitation, the present study shows for the first time that both IGF-1 and myostatin mRNA expression are reduced in the skeletal muscle of gastric cancer patients and suggests that the down-regulation of muscle IGF-1 and myostatin gene expression is an early event in the course of the

disease. Further studies are warranted to understand whether such alterations persist or if a different modulation in the expression of these genes may occur in advanced disease stages associated with greater body weight loss.

Disclosure statement

The authors declare no conflict of interest

Abbreviations:

IGF-1, Insulin-like growth factor 1; mIGF-1, isoform of IGF-1 in the skeletal muscle.

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Figure legends

- Figure 1** IGF-1 mRNA levels in the *rectus abdominis* muscle of gastric cancer patients.
- (A) Pattern of IGF-1 expression evaluated by semi-quantitative RT-PCR. Upper panel: data (means \pm SEM, n = 16) expressed as percentage of controls (C=0.46 \pm 0.14). Significance of the differences: *p<0.05 vs controls (n = 6). Lower panel: representative RT-PCR pattern; (B) IGF-1 expression evaluated by real-time PCR. Data are normalized fold expression (means \pm SEM; controls: n = 3; patients: n = 8). Significance of the differences: *p<0.05 vs controls.
- Figure 2** Myostatin mRNA levels in the *rectus abdominis* muscle of gastric cancer patients.
- (A) Pattern of myostatin expression evaluated by semi-quantitative RT-PCR. Upper panel: data (means \pm SEM, n = 16) expressed as percentage of controls (C=0.91 \pm 0.26). Significance of the differences: ***p<0.001 vs controls (n = 6). Lower panel: representative RT-PCR pattern. (B) Myostatin expression evaluated by real-time PCR. Data are normalized fold expression (means \pm SEM; controls: n = 3; patients: n = 8). Significance of the differences: ***p<0.001 vs controls.
- Figure 3** Expression of atrogin-1 and MuRF1 in the *rectus abdominis* muscle of gastric cancer patients.
- Upper panel: densitometric analysis. Data (means \pm SEM; n = 16) expressed as percentage of controls (Atrogin-1: C=0.91 \pm 0.26; MuRF1: C=0.33 \pm 0.14).

Significance of the differences: *** $p < 0.001$ vs controls ($n = 6$). Lower panel: representative RT-PCR pattern.

Table legends

Table 1: Characteristics of the subjects studied. Values are expressed as means \pm SEM; * $p < 0.05$ vs. controls.

Table 2: Oligonucleotide sequences used for semiquantitative reverse-transcriptase PCR (table 1A) and Real-time PCR (table 1B). The same oligonucleotides for IGF-1, Myostatin, Atrogin-1 and MuRF1 were used for both reverse transcriptase and Real-time PCR. The amplification protocols used for semiquantitative reverse-transcriptase PCR are indicated in table 1A.

Table 1.

	<u>Controls</u>	<u>Patients</u>
<u>Subjects</u>		
n	6	16
Age, years	62 ± 5.71	64 ± 2.75
<u>Type</u>		
Adenocarcinoma (n)	-	14
Lymphoma (n)	-	2
<u>Stage</u>		
I-II	-	12
III-IV	-	4
<u>Anthropometric parametrs</u>		
Percent weight loss	0.5 ± 0.04	5.3 ± 1.22
Body Mass Index	26.2 ± 2.08	28.7 ± 1.07
Serum total protein (g/L)	66.7 ± 1.84	64.8 ± 1.4
Serum albumin (g/L)	36.2 ± 1.59	35.1 ± 1
Serum creatinine (mg/dL)	0.94 ± 0.08	0.90 ± 0.02
Hemoglobin (g/dL)	14.2 ± 0.86	11.0 ± 0.85*
White cell count (x10 ³ /cmm)	6.9 ± 0.86	6.5 ± 0.4
Total lymphocyte count (x10 ³ /cmm)	2.41 ± 0.45	1.77 ± 0.2

Table 2.

A. Reverse transcriptase-PCR

<u>Gene</u>	<u>Primer sequence</u>	<u>Amplification</u>	<u>Cycles</u>
GAPDH (NM_002046.3)	FW: GGTGAAGGTCGGAGTCAACG RW: CAAAGTTGTCATGGATGACC	1' 94°C 1' 55°C 1' 72°C	24
IGF-1 (NM_001111283.1)	FW: CAGCAGTCTTCCAACCCAAT RW: CACGAACTGAAGAGCATCCA	30'' 94°C 1' 60°C 1' 72°C	35
Myostatin (NM_005259.2)	FW: TGGTCATGATCTTGCTGTAACCTT RW: TGTCTGTTACCTTGACCTCTAAAAACG	1' 95°C 1' 60°C 1' 72°C	35
Atrogin-1 (NM_058229.2)	FW: TCACAGCTCACATCCCTGAG RW: AGACTTGCCGACTCTTTGGA	1' 95°C 1' 58°C 1' 72°C	25
MuRF-1 (NM_032588.2)	FW: TGAGCCAGAAGTTTGACACG RW: TGATGAGTTGCTTGGCAGTC	1' 95°C 1' 58°C 1' 72°C	25

B. Real-time PCR

<u>Gene</u>	<u>Primer sequence</u>
GAPDH (NM_002046.3)	FW: CCACTCCTCCACCTTTGAC RW: ACCCTGTTGCTGTAGCCA
TBP (NM_003194)	FW: TGCACAGGAGCCAAGAGTGAA RW: CACATCACAGCTCCCCACCA

Figure 1

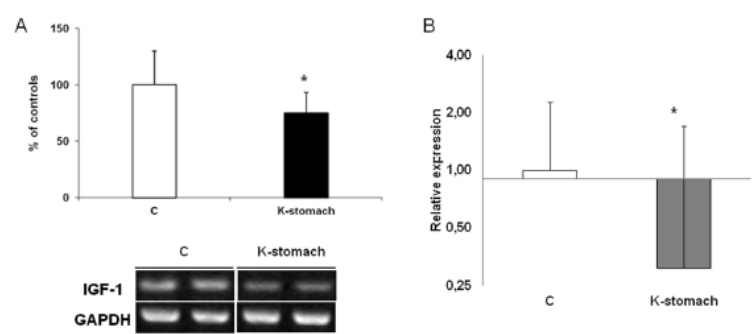


Figure 2

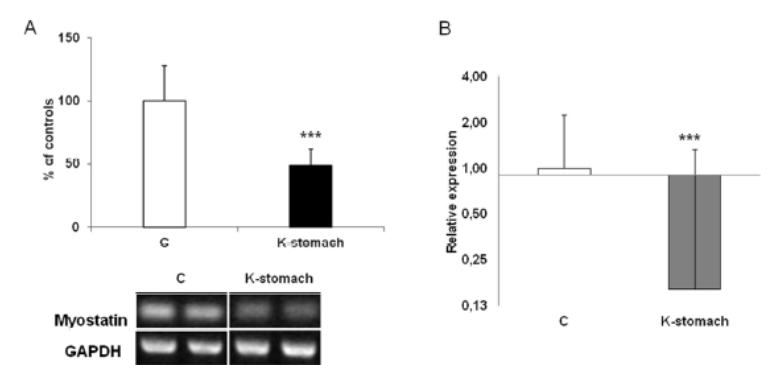


Figure 3

